

TITLE OF THE INVENTION

MraY GENE AND ENZYME OF *PSEUDOMONAS AERUGINOSA*

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not Applicable

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not Applicable

10 REFERENCE TO MICROFICHE APPENDIX

Not Applicable

FIELD OF THE INVENTION

15 This invention relates to the genes and enzymes involved in cell wall synthesis in bacteria.

BACKGROUND OF THE INVENTION

20 The pathway of peptidoglycan (PG) biosynthesis is both essential and unique to bacteria and the responsible enzymes are present in both Gram-negative and Gram-positive bacteria. Thus, inhibitors of these enzymes are likely to be broad spectrum and safe antibiotics. In fact, several enzymes in this pathway are molecular targets of naturally occurring antibiotics such as fosfomycin, cycloserine, β -lactams and vancomycin (Bugg & Walsh, 192 *Nat. Prod. Rep.* 9:199-215).

25 One enzyme intrinsic to the peptidoglycan biosynthesis is MraY. To date, no MraY sequences have been disclosed for *Pseudomonas aeruginosa*, an opportunistic pathogen causing infections in patients with burns or neutropenia. More serious is its involvement in respiratory tracts of cystic fibrosis patients.

30 It would be desirable to have polynucleotides and polypeptides encoding the MraY protein of *Pseudomonas aeruginosa* in order to further screen compounds for antibiotic activity against this enzyme catalytically active in the first step of the membrane cycle of peptidoglycan biosynthesis. Inhibitors of this enzyme would be particularly helpful in preventing the growth of *Pseudomonads* and other G+C rich bacteria.

Possession of this information would also greatly facilitate determinations as to the role of the encoded enzyme, Phospho-*N*-Acetylmurmoyl-Pentapeptide-Translocase, in the pathogenesis of infection and disease.

5 SUMMARY OF THE INVENTION

Polynucleotides and polypeptides of *Pseudomonas aeruginosa* MraY, an enzyme involved in bacterial cell wall biosynthesis, are provided. The recombinant MraY enzyme is catalytically active in the first step of the membrane cycle of peptidoglycan biosynthesis, transferring the *N*-acetylmuramic acid
10 pentapeptide to a bactoprenol phosphate carrier molecule. The enzyme is useful in *in vitro* assays to screen for antibacterial compounds that target cell wall biosynthesis. The invention includes the polynucleotides, proteins encoded by the polynucleotides, and host cells expressing the recombinant enzyme, probes and primers, and the use of these molecules in assays.

15 An aspect of this invention is an isolated polynucleotide having a sequence encoding a *Pseudomonas aeruginosa* MraY protein, or a complementary sequence. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In preferred embodiments the polynucleotide can be DNA, RNA or a mixture of both, and can be single or double stranded. In a most
20 preferred embodiment, the polynucleotide has a sequence shown in SEQ ID NO:1.

An aspect of this invention is a probe having a sequence of at least about 25 contiguous nucleotides that is specific for a naturally occurring polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein. Probes in
25 accordance with this description are useful for the specific detection of the presence of a polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein. In preferred embodiments, the probes of this aspect can have additional components including, but not limited to, compounds, isotopes, proteins or sequences for ready detection.

An aspect of this invention is a primer having a sequence of at least about 25 contiguous nucleotides that is specific for a naturally occurring
30 polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein. Primers in accordance with this description are useful in nucleic acid amplification-based assays for the specific detection of the presence of a polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein. In preferred embodiments, the primers of this aspect can have additional components including, but not limited to, compounds,
35 isotopes, proteins or sequences for ready detection.

An aspect of this invention is an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein, or a complementary sequence, and regulatory regions. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In particular
5 embodiments, the vector can have any of a variety of regulatory regions known and used in the art as appropriate for the types of host cells the vector can be used in. In a most preferred embodiment, the vector has regulatory regions appropriate for the expression of the encoded protein in gram-negative prokaryotic host cells. In other
10 embodiments, the vector has regulatory regions appropriate for expression of the encoded protein in gram-positive host cells, yeasts, cyanobacteria or actinomycetes. In some preferred embodiments the regulatory regions provide for inducible expression while in other preferred embodiments the regulatory regions provide for constitutive expression. Finally, according to this aspect, the expression vector can be derived from a plasmid, phage, virus or a combination thereof.

15 An aspect of this invention is a host cell comprising an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MraY protein, or a complementary sequence, and regulatory regions. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In preferred embodiments, the host cell is a yeast, gram-positive bacterium,
20 cyanobacterium or actinomycete. In a most preferred embodiment, the host cell is a gram-negative bacterium.

An aspect of this invention is a process for expressing a MraY protein of *P. aeruginosa* in a host cell. In this aspect a host cell is transformed or transfected with an expression vector including a polynucleotide encoding a *Pseudomonas*
25 *aeruginosa* MraY protein, or a complementary sequence. According to this aspect, the host cell is cultured under conditions conducive to the expression of the encoded MraY protein. In particular embodiments the expression is inducible or constitutive. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2.

30 An aspect of this invention is a purified polypeptide having an amino acid sequence of SEQ ID NO:2. Cellular extracts comprising a polypeptide having the above amino acid sequence are also included within the instant invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Nucleotide sequence (SEQ ID NO: 1) and the predicted amino acid sequence (SEQ ID NO:2) of *P. aeruginosa* MraY. The amino acid sequence (SEQ ID NO:2) is presented in a three-letter code below the nucleotide sequence (nucleotides 34 to 1113 of SEQ ID NO: 1).

DETAILED DESCRIPTION OF THE INVENTION

This invention provides polynucleotides and polypeptides of a cell wall biosynthesis gene from *Pseudomonas aeruginosa*, referred to herein as MraY.

10 The polynucleotides and polypeptides are used to further provide expression vectors, host cells comprising the vectors, probes and primers, and assays for the presence or expression of MraY.

Bacterial *mraY* encodes for phospho-*N*-acetylmuramoyl-pentapeptide translocase, an enzyme responsible for catalyzing the first step of the membrane cycle of peptidoglycan biosynthesis, transfer of the *N*-acetylmuramic acid pentapeptide to a

15 bactoprenol phosphate carrier molecule.

The *mraY* gene was cloned from *Pseudomonas aeruginosa*. Sequence analysis of the *P. aeruginosa mraY* gene revealed an open reading frame of 361 amino acids.

20 Nucleic acids encoding MraY from *Pseudomonas aeruginosa* are useful in the expression and production of the *P. aeruginosa* MraY protein. The nucleic acids are also useful in providing probes for detecting the presence of *P. aeruginosa* MraY.

As used herein, the following definitions apply:

25 The term "about" in the specification means within approximately 10-20% greater or lesser than particularly stated.

The term "polynucleotide" means a nucleic acid of more than one nucleotide. A polynucleotide can be made up of multiple polynucleotide units that are referred to by description of the unit. For example, a polynucleotide can comprise

30 within its bounds a polynucleotide(s) having a coding sequence(s), a polynucleotide(s) that is a regulatory region(s) and/or other polynucleotide units commonly used in the art.

The term "expression vector" means a polynucleotide having regulatory regions operably linked to a coding region such that, when in a host cell,

35 the regulatory regions can direct the expression of the coding sequence. The use of

expression vectors is well known in the art. Expression vectors can be used in a variety of host cells and, therefore, the regulatory regions are preferably chosen as appropriate for the particular host cell.

5 The term "regulatory region" means a polynucleotide that can promote or enhance the initiation or termination of transcription or translation of a coding sequence. A regulatory region includes a sequence that is recognized by the RNA polymerase, ribosome, or associated transcription or translation initiation or termination factors of a host cell. Regulatory regions that direct the initiation of transcription or translation can direct constitutive or inducible expression of a coding
10 sequence.

The terms "purified" and "isolated" are utilized interchangeably to stand for the proposition that the polynucleotide, protein and polypeptide, or respective fragments thereof in question have been removed from the *in vivo* environment so that they exist in a form or purity not found in nature. This, however,
15 is not mandated of cDNA as understood by one of ordinary skill in the art.

The term "substantially pure" with regard to a polynucleotide means it is obtained purified from cellular components by standard methods at a concentration of at least about 100-fold higher than that found in nature. A polynucleotide is considered essentially pure if it is obtained at a concentration of at least about 1000-
20 fold higher than that found in nature.

Polynucleotides

Polynucleotides useful in the present invention include those described herein and those that one of skill in the art will be able to derive therefrom following
25 the teachings of this specification.

An aspect of the present invention is a polynucleotide encoding a *MraY* protein of *Pseudomonas aeruginosa*. It is known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences that encode RNA
30 comprising alternative codons which code for the eventual translation of the identical amino acid. The present invention, thus, discloses codon redundancy which can result in different DNA molecules encoding an identical protein. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation.

A further aspect of the present invention is a cDNA encoding a MraY protein of *Pseudomonas aeruginosa*.

A preferred aspect of the present invention is an isolated nucleic acid encoding a MraY protein of *Pseudomonas aeruginosa*. A preferred embodiment is a
5 nucleic acid having the sequence disclosed in FIG. 1, SEQ ID NO:1.

The isolated nucleic acid molecule of the present invention can include a ribonucleic or deoxyribonucleic acid molecule, which can be single (coding or noncoding strand) or double stranded, as well as synthetic nucleic acid, such as a synthesized, single stranded polynucleotide.

10 Noncoding or antisense strands can be useful as modulators of the gene by interacting with RNA encoding the MraY protein. Antisense strands are preferably less than full length strands having sequences unique or specific for RNA encoding the polypeptide.

Also included in the present invention are polynucleotides that
15 hybridize to *P.aeruginosa mraY* sequences under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100
20 µg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10⁶ cpm of ³²P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would include
25 either a hybridization step carried out in 5 X SSC, 5 X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of
30 these reagents can be found in, e.g., Sambrook, *et al.*, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

Polypeptides

A preferred aspect of the present invention is a substantially purified form of the MraY protein from *Pseudomonas aeruginosa*. A preferred embodiment is a protein that has the amino acid sequence which is shown in FIG. 1, in SEQ ID NO:2.

Probes and Primers

Probes comprising full length or partial sequences of SEQ ID NO: 1 can be used to determine whether a cell or sample contains *P. aeruginosa mraY* DNA or RNA. A preferred probe is a single stranded antisense probe having at least the full length of the coding sequence of MraY. It is also preferred to use probes that have less than the full length sequence, and contain sequences specific for *P. aeruginosa mraY* DNA or RNA. The identification of a sequence(s) for use as a specific probe is well known in the art and involves choosing a sequence(s) that is unique to the target sequence, or is specific thereto. It is preferred that probes have at least about 25 nucleotides, more preferably about 30 to 35 nucleotides. The longer probes are believed to be more specific for *P. aeruginosa mraY* gene(s) and RNAs and can be used under more stringent hybridization conditions. Longer probes can be used but can be more difficult to prepare synthetically, or can result in lower yields from synthesis.

Examples of sequences that are useful as probes or primers for *P. aeruginosa mraY* gene(s) are Primer A (sense) 5'- TT CAT ATG CTC CTG CTG CTG GCC GAA TAC -3' (SEQ ID NO:3) and Primer B (antisense) 5'- TT GGA TCC TCA ACG CAG CTT CAA GGT G -3' (SEQ ID NO:4). Restriction sites, underlined, for NdeI and BamHI are added to the 5' ends of the primers to allow cloning between the NdeI and BamHI sites of the expression vector pET-11a. However, one skilled in the art will recognize that these are only a few of the useful probe or primer sequences that can be derived from SEQ ID NO:1.

Polynucleotides having sequences that are unique or specific for *P. aeruginosa* MraY can be used as primers in amplification reaction assays. These assays can be used in tissue typing as described herein. Additionally, amplification reactions employing primers derived from *P. aeruginosa* MraY sequences can be used to obtain amplified *P. aeruginosa mraY* DNA using the *mraY* DNA of the cells as an initial template. Many types of amplification reactions are known in the art and include, without limitation, Polymerase Chain Reaction, Reverse Transcriptase

Polymerase Chain Reaction, Strand Displacement Amplification and Self-Sustained Sequence Reaction. Any of these or like reactions can be used with primers derived from SEQ ID NO:1.

5 Expression of *MraY*

A variety of expression vectors can be used to express recombinant *MraY* in host cells. Expression vectors are defined herein as nucleic acid sequences that include regulatory sequences for the transcription of cloned DNA and the translation of their mRNAs in an appropriate host. Such vectors can be used to
10 express a bacterial gene in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells. Specifically designed vectors allow the shuttling of genes between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of
15 useful restriction enzyme sites, a potential for high copy number, and regulatory sequences. Expression vectors can include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

In particular, a variety of bacterial expression vectors can be used to express recombinant *MraY* in bacterial cells. Commercially available bacterial
20 expression vectors which are suitable for recombinant *MraY* expression include, but are not limited to pQE (Qiagen), pET11a (Novagen), lambda gt11 (Invitrogen), and pKK223-3 (Pharmacia).

Alternatively, one can express *mraY* DNA in cell-free transcription-translation systems, or *mraY* RNA in cell-free translation systems. Cell-free synthesis
25 of *MraY* can be in batch or continuous formats known in the art.

One can also synthesize *MraY* chemically, although this method is not preferred.

A variety of host cells can be employed with expression vectors to synthesize *MraY* protein. These can include *E. coli*, *Bacillus*, and *Salmonella*. Insect
30 and yeast cells can also be appropriate.

Following expression of *MraY* in a host cell, *MraY* polypeptides can be recovered. Several protein purification procedures are available and suitable for use. *MraY* protein and polypeptides can be purified from cell lysates and extracts, or from culture medium, by various combinations of, or individual application of
35 methods including ultrafiltration, acid extraction, alcohol precipitation, salt

fractionation, ionic exchange chromatography, phosphocellulose chromatography, lecithin chromatography, affinity (*e.g.*, antibody or His-Ni) chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and chromatography based on hydrophobic or hydrophilic interactions. In some instances, protein denaturation and refolding steps can be employed. High performance liquid chromatography (HPLC) and reversed phase HPLC can also be useful. Dialysis can be used to adjust the final buffer composition.

The following examples are presented in order to illustrate the instant invention.

EXAMPLE 1

General Materials and Methods

All reagents were purchased from SIGMA CHEMICAL CO., St. Louis, MO, unless otherwise indicated.

DNA manipulations reagents and techniques.

Restriction endonucleases and T4 ligase were obtained from Gibco-BRL. Agarose gel electrophoresis and plasmid DNA preparations were performed according to published procedures (Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989, Molecular cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory). Recombinant plasmids containing *P. aeruginosa mraY* were propagated in *E. coli* DH5 α (GIBCO-BRL, Rockville, MD) prior to protein expression in *E. coli* BL21(DE3)/plysS (NOVAGEN, Madison, WI). SDS-PAGE was performed with precast gels (NOVAGEN). DNA sequences were determined using an automated ABI PRISMTM DNA sequencer (PERKIN-ELMER ABI, Foster City, CA).

EXAMPLE 2

Cloning of *Pseudomonas aeruginosa mraY*

Genomic DNA from *P. aeruginosa* (strain MB4439) was prepared from 100 ml late stationary phase culture in Brain Heart Infusion broth (DIFCO, Detroit, MI). Cells were washed with 0.2 M sodium acetate, suspended in 10 ml of TEG (100 mM Tris, pH 7, containing 10 mM EDTA and 25% glucose) and lysed by incubation with 200 μ g of N-acetylmuramidase (SIGMA) for 1h at 37°C. Chromosomal DNA was purified from the cell lysate using a QIAGEN (Santa Clarita, CA) genomic DNA preparation kit following the manufacturers' protocol. Briefly, the cell lysate was treated with protease K at 50°C for 45 min, loaded onto an equilibrated QIAGEN genomic tip, and entered into the resin by centrifugation at 3000 rpm for 2 min. Following washing the genomic tip, the genomic DNA was eluted in distilled water and kept at 4°C. Approximately 50 ng genomic DNA was used as a template in PCR reactions to clone *mraY*.

Two oligonucleotide primers (GIBCO/BRL, Bethesda, MD) complementary to sequences at the 5' and the 3' ends of *P. aeruginosa mraY* were used to clone this gene using KLENTAQ ADVANTAGE™ polymerase (CLONTECH, Palo Alto, CA). The primer nucleotide sequences were as follows: 5'- TT CAT ATG CTC CTG CTG CTG GCC GAA TAC -3' (SEQ ID NO:3) and 5'- TT GGA TCC TCA ACG CAG CTT CAA GGT G -3' (SEQ ID NO:4). A PCR product representing *P. aeruginosa mraY* was verified by nucleotide sequence, digested with NdeI and BamHI, and cloned between the NdeI and BamHI sites of pET-11a, creating plasmid pPaeMraY. This plasmid was used for expression of the *mraY* gene in *E. coli*.

EXAMPLE 3

Overexpression and enzymatic activity of *Pseudomonas aeruginosa* MraY

mraY was cloned into the expression vector pET-11a (Novagen) as described above to create plasmid pPaeMraY. The pET-11a vector allows expression of authentic, non-fusion, proteins. The pET (Plasmids for Expression by T7 RNA polymerase) plasmids are derived from pBR322 and designed for protein over-

production in *E. coli*. The vector pET-11a contains the ampicillin resistance gene, and ColE1 origin of replication, in addition to T7 phage promoter and terminator. The T7 promoter is recognized by the phage T7 RNA polymerase but not by the *E. coli* RNA polymerase. A host *E. coli* strain such as BL21(DE3)pLysS is engineered to contain integrated copies of T7 RNA polymerase under the control of lacUV5 that is inducible by IPTG. Production of a recombinant protein in the *E. coli* strain BL21(DE3)pLysS occurs after expression of T7RNA polymerase is induced.

The pPaeMraY plasmid was introduced into the host strain BL21 DE3/pLysS (NOVAGEN) for expression of MraY. Colonies were grown at 37°C in 100 ml of LB broth containing 100 mg/ml ampicillin and 32 µg/ml chloramphenicol. When cultures reached a cell density of $A_{600}=0.5$, cells were pelleted and then resuspended in M9ZB medium (NOVAGEN) containing 1 mM IPTG. Cells were induced for 3 h at 30°C, pelleted at 3000g, and frozen at -80°C.

Cultures containing either the recombinant plasmid pPaeMraY or the control plasmid vector, pET-11a, were grown at 30°C and induced with IPTG. Lysates of cells transformed with pPaeMraY exhibited some 10.3 fold increase in MraY activity over uninduced cell lysates or induced lysates from cells containing the plasmid vector.

Assay for activity of MraY enzyme.

The MraY (translocase I) assay was performed using the butanol
 5 extraction method described by Brandish and coworkers (Brandish *et al.*, 1996 *J. Biol. Chem.* 271(13):7609-7614). The assay was performed at room temperature with assay components held at concentrations of: 100 mM TRIS, pH 7.5; 30 mM MgCl₂; 60.2 nCi [¹⁴C]UDP-MurNAc-pentapeptide (14 μM); 40 μM Decaprenol phosphate (Sigma Chemical Corp.); 0.15% Triton X-100 (w/v); and 100 mg/mL phosphatidyl
 10 glycerol (Sigma Chemical Corp.). Enzyme concentration was varied in order to obtain linear kinetics. Aliquots (50 μl) were removed at varying time points and transferred to a fresh tube containing 50 μl of 6M pyridinium acetate, pH4.2. The mixture was then extracted with 100 μl butanol and 50 μl water. After brief centrifugation, 80 μl of the top butanol layer was quantitated in a Packard TriCarb
 15 scintillation counter to determine the amount of Lipid I product produced.

Table 1

Specific activity of recombinant MraY
 from *P. aeruginosa*.

20

Recombinant MraY	Specific Activity	Fold Increase in Specific Activity
<i>P. aeruginosa mraY</i> in pET-11a vector	1428.2	10.3
Host <i>E. coli</i> cells containing pET-11a (empty vector – Control)	139.3	1